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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: A METHOD OF PRODUCING A FUNCTIONAL IMMUNOGLOBULIN SUPERFAMILY PROTEIN

(57) Abstract

The present invention relates to a process of producing a functional immunoglobulin superfamily protein, which has at least one disulphide bond when functional, the process comprising the steps of providing a bacterial cell comprising a gene coding for the protein, the gene is expressible in said cell, cultivating the cell under conditions where the gene is expressed, isolating the protein from the cell without reducing it, and subjecting the isolated protein to a folding treatment. Preferably, the immunoglobulin superfamily protein is selected from the group consisting of antibodies, immunoglobulin variable (V) regions, immunoglobulin constant (C) regions, immunoglobulin light chains, immunoglobulin heavy chains, CD1, CD2, CD3, Class I and Class II histocompability molecules, $\beta_2$microglobulin (β_2m), lymphocyte function associated antigen–3 (LFA–3) and FcγRIII, CD7, CD8, Thy–1 and Tp44 (CD28), T cell receptor, CD4, polyimmunoglobulin receptor, neuronal cell adhesion molecule (NCAM), myelin associated glycoprotein (MAG), P myelin protein, carcino–embryonic antigen (CEA), platelet derived growth factor receptor (PDGFR), colony stimulating factor–1 receptor, $\alpha\beta$–glycoprotein, ICAM (intercellular adhesion molecule), platelet and interleukins. Important embodiments of the invention is a stable peptide free MHC protein obtainable by a process of the invention and a kit comprising a MHC class I heavy chain and a β_2m allowing the recipient to produce and measure or detect a functional MHC class I protein to which a peptide, which is capable of binding to said MHC class I protein, can be added leading to the generation of a functional MHC class I protein.

A METHOD OF PRODUCING A FUNCTIONAL IMMUNOGLOBULIN SUPERFAMILY
PROTEIN

BRIEF DISCLOSURE OF THE INVENTION

5

The specificity and reactivity of the human immune system is governed by MHC (in hu-
mans HLA) molecules. The function of the HLA is to select and present antigenic peptides
to immune T cells. One could say that the immune system views the world through the
eyes of the MHC, and that any rational approach to immune manipulations must take

10 MHC into consideration. Such rational approaches should have many scientifical, practical
or clinical uses. To exploit these potentials we have devised a method which generates
recombinant MHC molecules of high purity in a novel, yet simple, robust and cheap man-
ner. These recombinant molecules are functionally fully active as peptide binders and T
cell stimulators. They can be generated in two distinct forms: a) as a fully mature, peptide

15 filled moiety, which is extremely stable and T cell stimulatory, and b) as a partially mature,
peptide free moiety ("empty" MHC molecules), which is reasonably stable and readily
peptide receptive. It should be noted that the latter result corrects the current misconcep-
tion that "empty" MHC molecules are non-existent, or at least extremely labile.

20 All other recombinant or non-recombinant MHC production methods known to us are
much more cumbersome and/or generates products of limited efficiency and purity. The
complexity and poor stability of MHC molecules makes it very difficult to generate pure
and peptide receptive MHC molecules and consequently, it is very difficult to generate
predetermined and pure peptide-MHC complexes. As natural molecules from natural

25 sources, the production of MHC molecules is tedious and of low yield. Furthermore, they
can only be purified by cumbersome methods leading to preparations where the MHC is
preoccupied by a large gamesh of different peptides and sometimes also contaminated by .
other MHC haplotypes the net result being grossly contaminated preparations.

30 Compounding the problems in generating MHC molecules Is the extremely polymorphism
of the MHC locus. In the human population more than 400 different HLA-A, HLA-B and
HLA-C alleles exist, and more that 200 HLA-D alleles exist. This diversity has an immu-
nological purpose, but is a practical obstacle to MHC production because many different
MHC's need to be generated and individually optimised, validated, characterised, stored

35 etc.

A recombinant expression system might have several advantages. One could potentially obtain a higher yield, an easy purification scheme, and the molecules could be labelled to homogeneity with one particular peptide. A major obstacle of such an approach, however,

5 is that the correctly folded MHC structure is a rather complicated structure consisting of three components (a heavy chain, a light chain (beta2-microglobulin, β_2m) and a peptide). The full stability of MHC is only acquired when all three components are together. In particular the heavy chain is extremely unstable in the absence of the two other components. Thus, it is difficult to produce, handle and store isolated heavy chain without the β_2m and

10 peptide, and it is therefore difficult to generate MHC molecules, which are readily available for binding of any peptide of the experimentors choice. Because of the limited stability, isolated MHC molecules rapidly aggregate and are lost leading to extremely poor final yields.

15 This patent application describes a general approach to generate recombinant MHC molecules in large amounts and of any allele specificity desired. The method also achieves for the first time truly empty, yet reasonably stable, MHC molecules - and the results show that the binding characteristics of these empty molecules deviate from those obtained with MHC generated in the past. It is a reasonable expectation that these empty

20 molecules are more relevant to physiological MHC binding since they reflect *de novo* binding, whereas previously used MHC molecules have reflected more artificial exchange reactions. In addition to the improved MHC quality, the novel production scheme is easy and robust, readily adaptable to most (probably all) MHC molecules, and it has a high yield of pure and fully functional MHC molecules. These recombinant molecules are ex-

25 tremely potent as the peptide binding activity of as little as 2-4 ng isolated MHC heavy chain can be detected, and the T cell binding capacity of as little as 150 ng peptide/MHC can be detected. They are extremely active with an affinity, which is similar to the one measured for natural occurring molecules, a much faster association rate - and fully available for binding (i.e. without endogenous peptides). We have been able to store these

30 molecules and maintain their activity over many months. The implications are both analytical and therapeutical.

Finally, the methods described have successfully been applied to other (non-MHC) molecules such as components of the CD3 complex. In fact, the methods disclosed would be

35 of use in any protein production scheme (be it recombinant or not, be it in prokaryotes or

in eukaryotes) where the protein at some point during the production is solvated by de-
naturation, or unfolding (the purpose could be to dissolve protein aggregates, to purify the
protein etc) necessitating a later renaturation/refolding step. Thus, the method may have a
very large field of application. In particular, we speculate that it might be useful for all
5 members of the immunoglobulin superfamily including antibodies, MHC and T cell recep-
tors. It might be useful for the production of all molecules containing at least one cysteine.

Background of the invention

10 The immune system can be viewed as one of natures bioinformatics systems. It evaluates
any substance that enters into our internal environment, determines its nature and de-
cides whether to take action against it. Proteins and peptides are the most important
means of obtaining and conveying such immune information. From this point of view,
MHC molecules are at the heart of the immune system. MHC molecules are sampling the
15 .entire protein metabolism for peptide information and makes this information available for
the central recognition unit of the immune system, the T cell. The current patent applica-
tion is related to a major undertaking to generate accurate methods to determine and pre-
dict the function of the MHC (a complete mapping of all human MHC reactivities). Com-
bining the growing genome databases of primary protein sequences of humans and para-
20 sites with the precise knowledge of how this immune molecule handles peptide informa-
tion will lead to new and powerful strategies for epitope prediction. This in turn will im-
prove the possibilities for directed and efficient immune manipulations. The ability to gen-
erate recombinant MHC molecules is enabling this large scale MHC/HLA mapping project
- and it does also have practical, clinical and scientifical uses of immediate commercial
25 interest as described below.

Introduction

The purpose of the immune system is to protect our body against microbial organisms
30 (e.g. bacteria, virus, parasites) - and perhaps against cancer. Virtually any threat can be
eliminated or neutralised by the immune system. To administrate such powers, the im-
mune system must know what to attack, and what not to attack; ideally, foreign matter
should be eliminated, while the body itself should be left unharmed. The true hallmark of
the immune system is therefore one of specificity i.e. the ability to discriminate between
35 various targets and in particular to distinguish between self and non-self. The specific im-

4

mune system consists of a large number of cells, or lymphocytes, with a major subdivision into B and T cells representing humoral (antibody) and cellular responses, respectively. Both cells use receptors, which in their genome are encoded in many bits and pieces allowing an enormous recombinatorial receptor diversity. Each B or T cell carries one, and

5   only one, of these receptors and can recognise a tiny part of the universe. All lymphocytes combined, however, can recognise the universe. The overall specificity of the immune system is generated, regulated and coordinated though processes controlling individual lymphocytes. Deleting, or inactivating, a lymphocyte clone removes the corresponding specificity from the repertoire. Activation and propagation of a lymphocyte clone enhances

10 the corresponding specificity - and allows the immune system to respond quickly and strongly should it be exposed to the same antigen again.

B and T cell specificities

15 B and T cells use entirely different mechanisms to recognise their targets. B cells recognise soluble antigens, and since they can secrete their receptors as antibodies, they can potentially interact with antigen throughout the fluid phase of the extracellular space. In sharp contrast, the T cell receptor is always membrane bound and it only recognises antigen, which is presented on the membrane of a so called antigen presenting cell (APC). In

20 other words, T cell recognition involves a direct physical interaction between two cells, a T cell and an APC. B and T cells also differ with respect to what they recognise. B cells can recognise organic substances of almost any kind, whereas T cells only recognise proteins (as a biological target, proteins are particularly important since they constitute the structural and functional basis of all life). B cells recognise the three dimensional structure of

25 proteins as illustrated by their ability to distinguish between native and denatured proteins. In contrast, T cells can not distinguish between native and denatured proteins. Early on, this led to the idea that T cells recognise altered proteins. We now know that this is true and that T cells only recognise antigenic peptides presented in association with MHC molecules on the surface of APC's. In general, cytotoxic T cells recognise short peptides

30 (8-11-mers) whose amino and carboxy-termini are deeply embedded within the MHC (i.e. the peptide length is restricted). In contrast, helper T cells tend to recognise longer peptides (13-30-mers or longer) with amino and carboxy terminal ends extending out of the MHC (i.e. the peptide length is unrestricted).

Immune responses and MHC restriction

T cells are of particular importance for the induction of immune responses since they de-
termine the reactivity and specificity of the entire immune system, including B cells. It is
5 therefore appropriate to focus our attention on these cells. T cells can only recognise a
given antigen, when it is presented in the context of particular MHC molecule. They are
"educated" during ontogeny and further activated during the first priming in processes de-
signed to develop T cells carrying receptors specific for a particular antigen-MHC combi-
nations. These T cells will subsequently only recognise the same exact same antigen-
10 MHC combination. This phenomenon is known as "MHC restriction". Another immune
phenomenon, that of "responder status", is also determined by the MHC. Individuals of
one MHC haplotype will respond to some antigens, and not to others. Other individuals
with other MHC haplotypes will respond differently. These two phenomena are of obvious
importance for any rational immune manipulation. As mentioned, we now know that they
15 are both controlled by MHC molecules. These molecules, which have specifically evolved
for the purpose of antigen presentation. Our current understanding of antigen presentation
can be summarised as follows. First, the foreign substance, the antigen, is taken up by
APC's. An intracellular pool of antigenic peptides is generated through proteolytic frag-
mentation of all the protein antigens available to the cell. This pool of peptides is offered
20 to the MHC molecules of the individual and sampled according to length and sequence;
some are bound, while others are ignored (the MHC is said to perform "determinant se-
lection". Subsequently, MHC molecules protect the selected peptides against further de-
gradation, transport them to the surface of the APC and display them for T cell scrutiny.

25 MHC and polymorphism

Two subtypes of MHC exist, MHC class I and II. These subtypes correspond to two sub-
sets of T lymphocytes: 1) CD8+ cytotoxic T cells, which usually recognise peptides pre-
sented by MHC class I molecules, and kill infected or mutated cells T cells, and 2) CD4+
30 helper T cells, which usually recognise peptides presented by MHC class II molecules,
and regulate the responses of other cells of the immune system. MHC class I consists of
a 43,000 MW transmembrane glycoprotein (the α chain) non-covalently associated with a
12,000 MW non-glycosylated protein (the β chain, also known as β_2-microglobulin). MHC
class II has an overall structure similar to MHC class I although the domain distribution is
35 different. Class II consists of two non-covalently associated transmembrane glycoproteins

of approximately 34,000 and 29,000 MW. The detailed structure of MHC class I and II molecules has been solved at the X-ray crystallography level (Björkman et al., 1987). The most interesting part of the MHC structure is the "upper" part which show a unique peptide binding groove consisting of two alpha helixes forming the walls of the groove and eighth
5 beta-pleated sheaths forming the floor of the groove.

The MHC is extremely polymorphic i.e. many different versions (alleles, allotypes) exist in the population, but each individual has only inherited one or two of these (one from the father and one from the mother). It is also polygenic i.e. several MHC encoding loci exist
10 in the genome allowing for simultaneous expression of several isotypes. Importantly, the majority of the polymorphic residues points towards the peptide binding groove affecting its size, shape and functionality (Matsumura et al., 1992). Peptide-MHC interactions are specific, albeit broad, allowing the binding of many unrelated peptides to each MHC allotype (Buus et al., 1987). The polymorphism dictates the specificity of peptide binding and
15 the biological consequence of this is that each individual in the population educates and shapes a unique T cell repertoire.

The generation of MHC specificity

20 Structurally, the peptide binding site of the MHC forms a groove, which can be subdivided into various pockets, A through F. The majority of the peptide-MHC binding energy involves main chain atoms of the bound peptide (including the termini for MHC class I); features which are common to all peptides (Matsumura et al., 1992). Only the minority of the binding energy involves peptide side chain atoms, however, these interactions are be-
25 lieved to explain the specificity of the MHC. This mechanism explains how the MHC achieves broad specificity, yet high affinity, peptide binding. Functionally, MHC achieves the broad peptide binding specificity through the recognition of "motifs" (Sette et al., 1987). A motif represents important structural requirements needed for peptide binding such as the presence and proper spacing of particular amino acids in anchor positions.
30 Considerable interest has focused on understanding how MHC specificity and motifs are generated, and on characterising the specificity of various MHC molecules. One of the ultimate goals of this effort is to be able to predict peptide binding. It follows from the MHC polymorphism (it has since been proven both structurally and functionally) that each MHC allotype has it own specificity characteristics. So far, these specificities can only be de-
35 scribed experimentally.

The description and prediction of MHC specificity

Two fundamentally different, but complementary, approaches are currently used to de-
5 termine the peptide binding specificity of MHC. One approach consists of sequencing the
peptides already bound to MHC molecules of a given allotype (Buus et al., 1988; Falk et
al., 1991), whereas the other approach consists of examining which peptides will bind to
the MHC (Buus et al., 1986; Olsen et al., 1994). Both approaches have advantages and
disadvantages. The sequencing method deals with naturally processed peptide-MHC
10 complexes, however, it arbitrarily assigns important vs. less important vs. non-important
residues and it cannot identify negatively interacting residues. It is thus best suited to
identify the most dominant of the positively interacting residues. The latter approach - the
direct binding method - is quantitative and it allows comparison of binders vs non-binders.
It can identify and quantitate both positively and negatively interacting residues. It is per-
15 haps not surprising that the direct binding method yields better predictability (about 70%
of the predicted peptides do indeed bind, than the sequencing method (about 30% suc-
cess) (Kast et al., 1994). It has been demonstrated that accurate predictions of peptide
binding require that the fine specificity of the MHC in question is known in detail (some-
times called extended motifs) (Parker et al., 1994; Rupert et al., 1993; Stryhn et al., 1996).
20 However, obtaining such detailed motifs is very labour and resource intensive. Presently,
to determine the fine specificity of every MHC molecule of interest large panels of pep-
tides biased towards particular sequences or motifs are routinely analysed (Parker et al.,
1994; Rupert et al., 1993). We have recently developed a peptide library based approach,
which yields a correct, unbiased and quantitative description of all functionally important
25 MHC binding residues (Stryhn et al., 1996). It is universal since many different MHC
molecules can be addressed with the same set of peptide libraries, and assays to test
binding can be developed for any MHC molecule. Conveniently, this approach signifi-
cantly reduces the experimental set-up and the subsequent data handling and should
therefore ease the complete mapping of all MHC class I specificities. This unbiased pep-
30 tide library based approach does also leads to improved peptide binding predictions
(Stryhn et al., 1996). The success of the predicting algorithm implies that MHC class I
binding can be viewed largely as the result of an array of independently acting sub-speci-
ficities.

The generation of recombinant MHC molecules

Bacteria as production vehicles for recombinant MHC molecules has been demonstrated
5   by others (Parker and Wiley, 1989). However, being packed in inclusion bodies within the
bacteria these MHC molecules have been unavailable for peptide binding. Strategies in-
volving complete denaturation and reduction of these inclusion bodies have been used to
extract and solubilize the recombinant MHC molecules (Parker et al., 1992; Parker et al.,
1992; Parker and Wiley, 1990). This has in turn necessitated the use of an *in vitro* re-
10   folding procedures in the presence of reducing/oxidising agents e.g. glutathion
(GSH/GSSG). Other components as L-arginine has been added to prevent aggregation
and misfolding. However, the *in vitro* folding faces a major problem in the generation of
correctly formed di-sulphide bridges. MHC class I heavy chains contains 4 (in some mole-
cules 5) cysteines. There are several possibilities for mis-paired di-sulphide bridges during
15   such re-folding. The general yield of functional MHC class I using this approach has been
reported to be low (about 10 - 20%), and of quite slow kinetics (Garboczi et al., 1992).

DETAILED DISCLOSURE OF THE INVENTION

20   The present invention relates to a process which has been invented in order to solve the
problem of having functional immunoglobulin superfamily proteins expressed in aggre-
gates such as inclusion bodies. In the process of the invention the functional protein may
consist of several protein subunits which are generated in the same cell or in different
cells. In the latter case, the functional proteins – which may very well be two different
25   kinds of proteins, e.g. a heavy chain of a MHC class I protein and a β2microglobulin - may
be combined at the time of folding or later.

In one embodiment, the invention relates to a process of producing a functional immuno-
globulin superfamily proteins, which have at least one disulphide bond when functional,
30   the process comprising the steps of

(i)   providing a bacterial cell comprising a gene coding for the protein, the gene is ex-
          pressible in said cell,

35   (ii)   cultivating the cell under conditions where the gene is expressed,

(iii)   isolating the protein from the cell under conditions, which do not change the
disulphide bonds generated by the cell, and, optionally, purifying the protein,

5 (iv)   subjecting the isolated protein to a folding treatment.

When the term "a" or "an" is used in the present specification and claims, this is meant to
be one or more, i.e. at least one.

10 By the term "a functional protein" is meant an immunoglobulin superfamily protein which is
capable of performing at least one of the functions attributed to said protein at least to a
substantial degree e.g. as assessed by an in vitro assay. By way of example, "a functional
MHC class I protein" is defined a protein which comprises a heavy chain, a light chain
(b2m) and a peptide. The heavy chain may be truncated in order to make it soluble in
15 aqueous solution. The peptide is a peptide which can be bound to the MHC protein in
question. Such peptides may be found by means of e.g. the direct binding method de-
scribed in Buus et al., 1986, and Olsen et al., 1994.

By "a functional MHC class II protein" is meant a protein which comprises a complex of
20 two heavy chains (an α and a β chain) and a peptide. The heavy chains may be truncated
in order to make the complex soluble in aqueous solution. The peptide is a peptide which
can be bound to the MHC protein in question. Such peptides may be found by means of
e.g. the direct binding method described in Buus et al., 1986, and Olsen et al., 1994.

25 Particularly preferred embodiments of the invention are processes wherein the MHC pro-
tein is a MHC class I protein selected from the group consisting of a heavy chain, a heavy
chain combined with a β2m, and a functional mature MHC class I protein; or a MHC class
II protein selected from the group consisting of an α/β dimer and an α/β dimer with a pep-
tide. One important aspect of the invention is a process wherein the produced MHC pro-
30 tein is obtained as a peptide free MHC protein.

By "a peptide free MHC class I protein" is meant a protein which comprises a heavy chain
associated with a light chain (b2m) but no peptide. A such protein may also be called an
"empty" MHC class I protein.
35

By "a peptide free MHC class II protein" is meant a protein which comprises a heavy chain complex but no peptide. A such protein may also be called an "empty" MHC class II protein.

5   The present invention is exemplified with reference to MHC class I proteins, but it envisaged that it may be possible in a similar manner to generate all immunoglobulin superfamily proteins (these are by definition disulfide bonded), i.e. a protein selected from the group consisting of antibodies, immunoglobulin variable (V) regions, immunoglobulin constant (C) regions, immunoglobulin light chains, immunoglobulin heavy chains, CD1, CD2,

10   CD3, Class I and Class II histocompatibility molecules, β2microglobulin (β2m), lymphocyte function associated antigen-3 (LFA-3) and FcγRIII, CD7, CD8, Thy-1 and Tp44 (CD28), T cell receptor, CD4, polyimmunoglobulin receptor, neuronal cell adhesion molecule (NCAM), myelin associated glycoprotein (MAG), P myelin protein, carcinoembryonic antigen (CEA), platelet derived growth factor receptor (PDGFR), colony stimulating factor-

15   1 receptor, αβ-glycoprotein, ICAM (intercellular adhesion molecule), platelet and interleukins. The present inventors have already provided data with respect to several MHC class I molecules, β2microglobulin, MHC class II molecules, T cell receptor CD3 chains gamma and epsilon.

20   Cloning of cDNA encoding the various proteins of interest follows standard procedures e.g. as described in Molecular Cloning (Sambrook, Fritsch and Maniatis, Cold Spring Harbor Press, 1989). Briefly, cDNA is synthesised from appropriate cell lines using commercial cDNA synthesis kits (in casu from Pharmacia). For human work the cells would be derived from the panel of HLA expressing EBV transformed human B cell lines from the

25   12th International Histocompatibility Workshop Cell Lines Panel Database ("HLA: Genetic diversity of HLA. Functional and Medical Implication", Ed. Dominique Charron, EDK press, 1997, or see http://www.icnet.uk/axp/tia/marsh/ihw.html). For HLA-A*0201 an appropriate cell line would be the IHW 9012. The nucleotide sequence corresponding to the desired MHC/HLA molecule can be found at http://www.anthonynolan.com/HIG/index.html, or at

30   http://www.ncbi.nlm.nih.gov. Using this sequence information oligonucleotide primers can be designed to amplify by the polymerase chain reaction the coding region encompassing amino acids 1 - 274 of the mature MHC/HLA molecule from the appropriate cDNA. A relevant forward and backward primer set for the purpose of amplifying HLA-A*0201 and insert it into the NcoI and HindIII restriction sites of the pET28a expression vector (Nova-

35   gen, see http://www.novagen.com/vectfram.html. The ligated product is transformed into

the bacteria TOP10F' and selected for kanamycin resistance overnight. Several clones are picked and their plasmids prepared by Wizard miniprep (Promega). The plasmids are used as templates in a polymerase chain reaction using the cloning primers and the amplificate is analysed by electrophoresis in agarose and Ethidium bromide stained. Plas-

5 mids which lead to amplificates of the appropriate size is sequenced (ABI 310 sequencer) to identify clones, which contain the desired sequence. These clones are secured and used for the subsequent production. A similar strategy can be used to clone any gene of interest.

10 It is particularly preferred that the protein is a vertebrate protein, e.g. a human, a murine, a rat, a porcine, a bovine, or an avian protein.

In another embodiment, the invention relates to a process of producing a plurality of functional proteins, where at least one of the proteins is the immunoglobulin superfamily, and

15 the plurality of proteins when functional contains at least one intramolecular or intermolecular disulphide bond, the process comprising the steps of

(i)   providing a bacterial cell comprising a plurality of genes coding for one protein each, all genes being expressible in said cell,

20

(ii)   cultivating the cell under conditions where the genes are expressed,

(iii)   isolating the proteins from the cell under conditions which do not change the disulphide bonds generated by the cell, and optionally, purifying them,

25

(iv)   subjecting the isolated proteins to a folding treatment.

In this embodiment, the protein can be a fusion protein or may be two separate proteins, i.e. co-expressed proteins.

30

A further embodiment relates to a process of producing a functional immunoglobulin superfamily protein, which has at least one disulphide bond when functional, the process comprising the steps of

(i)   providing a cell comprising a gene coding for the protein, the gene is expressible in
         said cell, the protein being expressed as an aggregate

(ii)   cultivating the cell under conditions where the gene is expressed,

5

(iii)   isolating the protein aggregate from the cell under conditions which do not change
         the disulphide bonds generated by the cell, and optionally, purifying it,

(iv)   subjecting the isolated protein to a folding treatment.

10

A denatured protein may be present in many different conformations – it does not have a
distinctive conformation - whereas a folded protein in aqueous solution is present in one
or a few distinct conformations. One of the essential features of this invention is that it
avoids the conventional solvation of the protein aggregate effected by denaturation under

15 reducing conditions which leads to a completely unfolded protein. Subsequent refolding in
order to generate a correctly folded protein is complicated by the requirement for recrea-
tion of the correct disulphide bonds. The method of the invention is different in that it has
surprisingly been found that the proteins present in the aggregates e.g. the inclusion
bodies seem to be present in a functional form having correct disulphide bonds and the

20 task thus is to have them solubilized without breaking the disulphide bonds. The present
inventors have found that the denaturing solvation of the protein must be performed under
non-reducing conditions without altering of the redox state of the protein. Using denatured
proteins with correct disulphide bonds leads to a simplification of the refolding process
which may now be as simple as dilution of e.g. urea without adding a redox couple. The

25 folding may, however, be assisted by other proteins such as chaperones; in case of MHC
class I it can be assisted by β2m and/or peptide. Further, the folding treatment according
to the invention can for certain proteins, e.g. MHC, be performed essentially in the ab-
sence of a redox couple such as GSSG/GSH.

30 The isolation may be performed by disrupting the cell, separating the aggregates such as
inclusion bodies (e.g. by centrifugation), optionally washing, extracting the aggregates
(e.g. inclusion bodies) in a denaturing agent (e.g. urea or guanidine-hydrochloride, or by
other methods known by a person skilled in the art) leading to extraction of soluble pro-
tein. This is a schematic outline of the isolation process of (iii) which may be modified or

35 followed e.g. by a purification step as will be evident to the person skilled in the art. This is

a particularly convenient time, for MHC molecules it is actually preferred to add a step of
purification since many non-covalent associated molecules including oligo-peptides can
be removed. Such purification may be ion-exchange chromatography, size exclusion
chromatography, affinity chromatography, hydrophobic interaction, precipitation, filtration,
5 centrifugation and other methods known by the person skilled in the art.

The folding is initiated by diluting the denaturing agent (e.g. urea) to a point which leads to
folding of the protein. Preferably, the folding step in the process of the invention (iv) is
performed in an aqueous medium which may comprise at least one buffer compound. The
10 protein may then be subjected to a purification procedure as described above.

In the process of the invention a cell which comprises a gene coding for a heterologous or
homologous protein, which gene is expressible in said cell, may be any cell. Preferably,
the cell is selected from the group consisting of a bacterial cell, a fungal cell, a yeast cell,
15 an animal cell and a plant cell. More preferably, the cell is a bacterial cell selected from
the group consisting of a gram positive bacterium and a gram negative bacterium. In a
presently preferred embodiment the gram negative bacterium is E. coli including a strain
BL21 or a derivative thereof or a strain XA90 or a derivative thereof.

20 It is contemplated that another useful cell could be a cell which is genetically modified to
have a less reducing intracellular environment than a non-modified cell of the same strain,
e.g. the cell has been modified to have a reduced or lacking activity of a thioredoxin re-
ductase or an enzyme having a similar effect on the sulfhydryl reducing potential of the
cytoplasm, such as a trxB-mutant. Another useful strain may be a strain which is capable
25 of biotinylating the protein, i.e. which is capable of biotinylating a protein which has a bio-
tinylating sequence. The protein may be modified in vivo or in vitro, e.g. phosphorylated,
glycosylated, acetylated, amidated or modified in any other appropriate way.

The expressed protein may be located intracellularly, periplasmatically or extracellularly.
30
The insertion of the gene coding for the functional protein is carried out by any conven-
tional technique for the introduction into a cell of nucleotide sequences, e.g. by transfor-
mation, transfection, or transduction. The gene can be inserted into the chromosome of
the host cell typically by means of transposome or by a recombination event, or it can be

introduced episomally by means of appropriate vectors. Suitable vectors for such pur-
poses includes the pET vectors, such as the T7 promoters.

It will be appreciated that the gene can be introduced into the host cell alone or in combi-
5 nation with further nucleotide sequences including sequences that regulate the expression
of the gene such as promoter sequences, sequences regulating the promoter, enhancer
sequences, sequences coding for repressor substances including antisense RNA, or ter-
mination sequences. In order to enhance the amount of protein produced multiple copies
of the gene coding for the functional protein can be introduced into the host cell. It is also
10 possible to introduce sequences coding for chaperone proteins or sequences regulating
the expression or functionality of native chaperone proteins, or sequences coding for gene
products that result in glycosylation of the functional protein. The promoter may be con-
stitutive or inducible.

15 The process according to the invention will be advantageous in one or more aspect of
protein product. It may be that the yield of functional protein produced according to the
process relatively to the yield of functional protein obtained under essentially similar con-
ditions but where step (iii) is performed under conditions which do change the disulphide
bonds generated by the cell, is increased by at least 10%, such as at least 20%, at least
20 40%, at least 50%, at least 70%, or at least 100%. Alternatively, it may be that the speed
of the process of the invention compared to when step (iii) is performed under conditions,
which do change the disulphide bonds generated by the cell, is at least 10% faster, such
as at least 20%, at least 40%, at least 50%, at least 70%, or at least 100%. It is contem-
plated that the increase of speed may in fact be as much as 2 fold, 5 fold, 10 fold, 100 fold
25 or 10000 fold increased. In a presently preferred embodiment, the speed is increased by
at least 50 fold. Finally, it may be that the purity of the functional protein produced ac-
cording to the process relatively to the purity of the resulting functional protein obtained
under essentially similar conditions but where step (iii) is performed under conditions,
which do change the disulphide bonds generated by the cell, is increased by at least 10%,
30 such as at least 20%, at least 40%, at least 50%, at least 70%, or at least 100%.

With reference to the examples, in particular the MHC protein A2, the folding efficiency
may be at least 40%, whereas the MHC protein Db has a folding efficiency which is con-
sidered to be even higher, i.e. at least 50%. The % is measured on the active protein im-
35 mediately after the folding compared to the amount of input protein in question in the

folding process. It is contemplated that when the process according to the invention has been optimised with regard to the protein in question, then at least 25% of the produced immunoglobulin superfamily protein is obtained in a functional form.

5 Preferably, the protein comprises no unpaired Cysteine residues. However, within the scope of the invention is an embodiment wherein the protein comprises 1 unpaired Cysteine residue. The protein may comprise at least 2, such as at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or at least 20

10 Cysteine residues. Preferably, the protein comprises an even number of Cysteine residues. Most likely, the protein is having at most 20, such as at most 14, at most 10, at most 8, at most 5, at most 4, at most 3, or at most 2 Cysteine residues. The protein is preferably capable of having at least 1, such as at least 2, at least 3, at least 4, at least 5, or at least 6 disulphide bonds. Most likely, the protein is capable of having at most 20, such as

15 at most 15, at most 10, at most 8, at most 5, at most 4, at most 3, or at most 2 disulphide bonds.

In a preferred embodiment the gene is a derivative of a naturally occurring gene. The derivative may be obtained by substituting at least one codon which is used more frequently

20 by the host cell than the one originally present where the codon codes for the same amino acid. Generally, the gene is under control of a regulatory DNA sequence not naturally associated with the gene. However, it may also under control of its own promoter. In a presently preferred embodiment the bacterial cell is transformed with an expression vector selected from the group consisting of pET vectors, e.g. T7 promoters. Other vectors will

25 be evident to the person skilled in the art.

A most important aspect of the invention is a stable peptide free MHC protein which is obtainable by a process according to the invention. A stable peptide free MHC protein has not previously been generated by any of the methods within the art. Although it has been

30 claimed (Matsumura et al., 1992) that empty molecules can be obtained from TAP deficient eucaryotic cells such as T2 or insect cells, peptides have been extracted and characterised from such preparations (Wei & Cresswell, 1992; Henderson et al., 1992), i.e. these preparations are not truly empty). One of the uses of the stable peptide free MHC protein is it provides for a highly efficient production of pure homogenous peptide-MHC

35 complexes. By the term "stable" is meant that the heavy chain in isolated form in urea can

be stored for at least 3 months at −20°C at 50% glycerol. The half-life of the complex is actually more than six months. The stability of the functional MHC complex in aqueous solution, i.e. the heavy chain and β2m in 1:1 is being investigated. However, it is known that the half-life of the heavy chain in the presence of an excess of β2m is stable in aque-

5   ous solution is about 2 days at 4°C.

Another important aspect of the invention is a kit comprising a MHC class I heavy chain and a β2m allowing the recipient to produce a functional MHC class I protein to which a peptide, which is capable of binding to said MHC class I protein, can be added leading to

10 the generation of a functional MHC class I protein. The MHC proteins will preferably be produced by the method according to the invention. In one embodiment, the kit comprises reagents that will allow the end-user to determine the binding of any peptide of his/her choice using detection systems such as enzyme linked immuno sorbent assay (ELISA), radio immuno assay (RIA), or others know to the person skilled in the art. In another em-

15 bodiment, the kit comprises an oligomerization of MHC proteins, such as two, three, four or more. In a specific embodiment, the kit comprises a further reagent added as a marker making the kit suitable for diagnostic purposes. The marker is preferably selected from the group consisting of fluorochromes, enzymes, chemiluminescense, and radioactive markers.

20

A preferred use of the process of the invention is in the manufacturing of MHC, in particular peptide free MHC molecules. A preferred use of a stable peptide free MHC protein is in analysis of the effect of changing an amino acid in the MHC on the binding specificity of said MHC as assessed by an analysis using a peptide library approach be it a synthetic

25 or recombinant library. Such a combination of MHC point mutations followed by specificity analysis by peptide libraries constitutes a novel approach to the examination of structure-function relationships. Further uses of the invention are described in the following.

The use of recombinant MHC I molecules

30

a) Analysis/diagnostics (in particular by ELISA and/or FACS)

a.1) Quantitation of peptide-MHC interaction. It is of considerable interest to measure the interaction between peptide and MHC. Any putative T cell epitope should be checked for

35 MHC binding, preferably in a quantitative assay. Current methodology for measuring such

interactions is hampered by poorly controlled assay systems, the lack of empty MHC molecules and/or the requirement for labelling at least one of the components. Empty molecules are highly active and easily adaptable to highly controlled biochemical assay systems of the RIA type (Buus et al, 1995). Empty molecules are also adaptable to detec-
5 tion by an ELISA approach. In a preferred embodiment, this ELISA involves pan-specific anti-MHC antibody capture and pan-specific anti-MHC antibody detection allowing highly sensitive, quantitative, non-radioactive detection. Other designs of the ELISA (e.g involving affinity tags) are known to the person skilled in the art.

10 a.2) Enumeration of specific T cells for quantitative and qualitative characterisation of T cell populations. Mark Davis and coworkers in 1996 (Altman et al., 1996) demonstrated that recombinant, pure peptide-MHC class I complexes could be generated with an added biotinylation signal attached to the C-terminal of the heavy chain. After an enzymatic biotinylation process these complexes could be tetramerized with Phycoreythin labelled
15 Streptavidin. These multimerized, labelled peptide-MHC class I complexes could subsequently be used to label T cells in a peptide-specific, MHC I-restricted manner. The stability of the T cell receptor for peptide-MHC I complexes is generally thought to be too low to effect a stable biochemical binding. However, after tetramerization, the avidity of the multimerized complexes is sufficient to effect bichemical binding. Thus, such tetramers
20 can be used to count by fluorescence activated cell sorter (FACS) analysis of the number of T cells in any given cell suspension. It has subsequently been shown that older methods for counting specific T cells, limiting dilution analysis, is grossly incorrect and underestimates the number of specific T cells. Thus, it has become imperative both from a scientific point of view, but also from a publication point of view, to do "tetramer" analysis.
25 The Davis method has two major problems: it is difficult to produce large amount of pure MHC I molecules, and the biotinylation process is expensive, cumbersome, and in particular, it requires extended incubation at 37°C. Many peptides do remain associated to the MHC I for the time of this latter incubation, and this may explain the variable result even within the same laboratory. The present patent application discloses a method
30 whereby peptide-MHC I complexes can be generated in a fast and efficient process leading to a minimum post-complex formation clean-up. It is envisioned that the MHC I molecules produced according to the present method will be amenable to transport and storage in a way that allows the generation of a commercial kit, which would further allow the final peptide-MHC I complex production to be done in the any non-expert laboratory using
35 any relevant peptide of the end-users choice. In contrast, previous ways of producing

MHC I would require considerable protein and molecular biology knowledge and experience. Finally, many better ways of tagging the MHC I than the enzymatic biotinylation process can be envisioned (Gallimore et al., 1998; Walter et al., 1998).

5   a.3) Enumeration of specific T cells enabling immune manipulations to be accurately monitored. Any immune manipulation (e.g. vaccination) is in need of an accurate and specific monitoring. The above "tetramer" technology is at this time the golden standard of scientific, clinical and commercial evaluation of immune manipulation.

10 b) Scientifically

b.1) Functional and structural determination of the specificity of MHC I molecules. MHC I molecules are central players in the generation of all T cell mediated responses. Considerable efforts are aimed at understanding the function and specificity of MHC I molecules.
15 For all these purposes one needs access to functionally active MHC I molecules. Producing MHC I molecules from natural sources have several serious draw-backs (cumbersome, expensive production, yet it yields small amounts of impure MHC I). The method disclosed here allows for an easy, fast and highly efficient production of peptide-MHC I complexes. Previous methods, which were used to produce peptide-MHC I complexes
20 included a step where an excess of peptide was offered to the MHC I under refolding (Garboczi et al., 1992). Thus, the resulting complexes were preoccupied with peptide and therefore not readily available for de novo peptide binding. According to the method disclosed here, the MHC I molecules produced can immediately be used for binding of peptide, and will therefore be useful for any analysis including, specificity analysis, of peptide
25 binding. Combining the recombinant MHC I molecules with the recently published peptide library approach (Stryhn et al., 1996), one could examine in detail the specificity of any MHC I molecule including mutated MHC I molecules. It should be stressed that such detailed analysis also leads to improved peptide binding predictions (Stryhn et al., 1996).

30 b.2) Functional and structural determination of the specificity of T cells. Peptide-MHC I complexes can be generated fast, pure and efficiently by the disclosed method. Such complexes can be used to analyse the structure of the T cell receptor in interaction with said peptide-MHC I complex, and using peptide variants and or MHC I variant it will also be possible to perform a functional analysis of the T cell receptor specificity.

35

b.3) Specific T cell manipulation (induction or blocking). The peptide-MHC I complexes disclosed here will be able to interact with the T cell receptor of a given target cell. In order to stimulate the cell its T cell receptors must be cross-linked. Thus, one can expect that polymerised peptide-MHC I complexes (say "tetramers") might stimulate appropriate

5   peptide-specific, MHC I-restricted T cells, whereas soluble peptide-MHC I complexes might block the same cells.

b.4) Monitoring the specific effect of immune manipulations (e.g. vaccinology). Any investigation on how to manipulate the T cell immune system will - until further improvements in

10 technology - depend on the existence of the "tetramer" (or tetramer-like) technology. The ability to induce specific predetermined responses can be accurately and easily determined, and by modern FACS analysis even further analysed into which subpopulations are affected and how much. The tetramer technology will therefore be essential for future developments in immune manipulations

15

b.5) Purifying specific T cells. Tetramers should allow the efficient purification of peptide-specific, MHC I-restricted T cells. By way of example, if the tetramers through their biotin were coupled to paramagnetic beads it would be relatively straightforward to purify the corresponding specific T cells using a magnet. The specific T cells could then be eluted

20 off and used for analysis or expanded and used for further immune manipulations (e.g. adoptive T cell transfer). This would constitute a vast improvement compared to present cloning technology which is extremely cumbersome and slow.

c) Therapy

25

c.1) Vaccine development. The effects on vaccine development can be crudely subdivided into a direct and an indirect effect. A direct effect of the disclosed technology would be the therapeutic application of the principle mentioned in b3, where a highly specific and very efficient activation of specific T cells (and subsequently of the immune system) is en-

30 visioned based on isolated, peptide loaded MHC I molecules administered in a stimulatory way (cross-linked as "tetramers", on beads etc.). An indirect effect would be caused by the improved identification of vaccine candidates that will be the result of the disclosed technology. By enabling a large scale detailed analysis of all human MHC I molecules it will improve predictions of pathogen derived peptides capable of binding to MHC, and al-

35 low an easy validation of such predictions (MHC specific epitope analysis).

c.2) Treatment of autoimmune diseases. The effects on the treatment of autoimmune dis-
eases can be crudely subdivided into a direct and an indirect effect. A direct effect of the
disclosed technology would be the therapeutic application of the principle mentioned in
5 b3, where a highly specific blocking of specific T cells (and subsequently of significant
parts of the immune system) is envisioned based on isolated, peptide loaded MHC I
molecules administered in a non-stimulatory way (i.e. as soluble, non-cross-linked com-
plexes). An indirect effect would be caused by the improved identification of autoimmune
disease inducing candidates that will be the result of the disclosed technology. By ena-
10 bling a large scale detailed analysis of all human MHC I molecules it will improve predic-
tions of self-protein derived peptides capable of binding to MHC, and allow an easy vali-
dation of such predictions.

c.3) Purification of T cells for adoptive transfer. As detailed in b5. T cells can be specifi-
15 cally labelled using "tetramers" - and therefore also sorted (i.e. by magnetic beads) lead-
ing to the preparation of pure T cell populations of predetermined specificity. Such T cell
populations can then be expanded and used e.g. for adoptive transfer against infectious
or oncogenic diseases.

20 c.4) Treatment of cancer. Many cancers are associated with mutated oncogenes/tumor
suppressor genes, or with dysregulated oncogenes/suppressor genes. The resulting
change in the cellular metabolism can be detected by the immune system. The are now
examples of dysregulations leading to an altered level of an otherwise completely normal
self protein. Yet, the altered level of the self-protein renders it immunogenic and tumor-
25 rejection has been demonstrated in such cases (Vierboom et al., 1997). Mutations and
dysregulation can be detected as exemplified, but not limited to, by genetic analysis (e.g.
"single strand conformational polymorphism", "selective polymerase chain reaction" or
"differential display") or protein analysis (mass spectrometry driven proteome analysis).
Any such identified protein can be subjected to the above described MHC specific epitope
30 analysis and specific treatment of the cancer can be attempted as detailed in c1 and c3.

LEGEND TO FIGURES

Figure 1:

5   Production of recombinant HLA-A*0201. XA90 cells encoding truncated A2 (1-275) was
induced with 0.4 mM IPTG. Production was analysed in 15% SDS-PAGE at reducing
conditions.
Lane 1: markers as indicated. Lane 2: cellular proteins before induction. Lane 3: cellular
proteins 3 hours after induction. Lane 4: recombinant HLA-A*0201 after purification using
10 anion-exchange chromatography.

Figure 2:

Anion-exchanged fractions with recombinant HLA-A*0201 heavy chain from urea solubi-
15 lised inclusion body proteins analysed in a 15% SDS-PAGE at non-reducing condition.
Fractions corresponding to purified peptide binding monomers (fraction numbers indicated
at the top, compare with figure 4) were analysed. It is shown that recombinant HLA-
A*0201 migrates as two distinct proteins about 31-32 kD; protein band 2a and 3, both with
intact disulfide bonds.
20
Figure 3:

SDS-page analysis of purified recombinant HLA-A*0201 molecules in presence or ab-
sence of DTT. Lane 1: Highly purified and functional HLA-A*0201 heavy chain resulting
25 from a folding by dilution process (non-reduced). Lane 2 and 3: Anion exchanged HLA-
A*0201 heavy chain at non-reducing condition (band 2a and 3). In lane 3 it is shown that
both proteins bands are partly reduced to band 1 by the reductant present in lane 4.
Lane 4: Anion exchanged HLA-A*0201 heavy chain at reducing condition revealing pro-
tein band 1, which migrates slower than protein band 2a and 3.
30
Figure 4:

Anion exchanged fractions analysed for amount of eluded proteins and corresponding ca-
pacity of peptide binding. The concentrations were determined by BCA and measured at
35 OD562 (full line). The same fractions were also tested for peptide binding. About 30% of

the total amount of protein applied to the column do not bind (basic or neutral charged proteins). These proteins correspond to the bacterial background proteins co-purified with the inclusion bodies. Recombinant HLA-A*0201 molecules eluded with about 200 mM NaCl and were identified by peptide binding analysis (stippled line) and SDS-PAGE

5   analysis (figure 3).

Figure 5:

Immunoprecipitation of folded recombinant HLA-A*0201 molecules using specific anti-

10   bodies.

Complexes of recombinant HLA-A*0201 heavy chain, b2m and radiolabelled peptide were incubated with monoclonal antibodies against either HLA-A*0201, H-2K^k or H-2D^b molec-ules at 4°C. Subsequently, protein A was added to precipitate immune complexes. The

15   precipitate was washed repeatedly and counted for radioactivity. Only HLA-A*0201 spe-cific antibodies BB7.2 and W6/32 could precipitate the recombinant HLA-A*0201 bound radiolabelled peptide. There was no measurable interaction between recombinant HLA-A*0201 and antibodies with irrelevant specificity.

20   **Figure 6:**

Peptide binding to recombinant HLA-A*0201 heavy chain.

Increasing doses of recombinant HLA-A*0201 heavy chains were incubated with b2m

25   (1 μM) and tracer amount of radiolabelled peptide (2 nM) for 4 hr. at 18°C. The degree of complex formation was determined by G25 spun-column chromatography.

Figure 7:

30   The affinity of peptide interaction with recombinant HLA-A*0201.

5 nM recombinant HLA-A*0201 was incubated with trace amounts of radiolabelled peptide and b2m (1 uM) and increasing concentrations of unlabeled peptides with specificity to HLA-A*0201 and H2-Kk, respectively. The reactions were incubated for 4 hr. at 18°C, and

35   the degree of complex formation was determined by G25 spun-column chromatography.

The bound ligand concentration vs. the ratio of bound and free ligand was plotted to obtain a Scatchard plot (insert).

Figure 8:

5

The b2m dependent peptide binding to recombinant HLA-A*0201.

Recombinant HLA-A*0201 heavy chains were incubated with tracer amount peptide and increasing concentrations of human and mouse b2m as indicated. The reactions were in-
10 cubated for 4 hr. at 18°C, and the degree of complex formation was determined by G25 spun-column chromatography.

Figure 9:

15 Increasing doses of recombinant HLA-A*0201 heavy chains were incubated with radio-labelled human b2m in presence or absence of specific peptides (10 uM) for 4 hr. at 18°C. The reactions were incubated for 4 hr. at 18°C, and the degree of complex formation was determined by G50 spun-column chromatography.

20 **Figure 10:**

The dissociation of b2m from recombinant HLA-A*0201 complex. Spun column purified complexes of heavy chain and radiolabelled b2m with or without peptide were mixed with 3 uM unlabeled b2m and incubated for the time indicated at 4°C. The degree of dissocia-
25 tion was determined by Sephadex G50 spun column chromatography.

Figure 11:

Tetramer staining as assessed by FACS analysis. The cells analysed are CD8 positive T
30 cells either from mice carrying a transgene for a T cell receptor specific for the KAVYN-FATM peptide in association with the H-2D[b] or from non-transgene control H-2D[b] mice. The analysis was performed with phycoerythrin-streptavidin generated tetramers involving either the relevant KAVYNFATM peptide in complex with recombinant H-2D[b] and biot-inylated b2m, or with the irrelevant FAPGNYPAL peptide in complex with recombinant H-
35 2D[b] and biotinylated b2m. The FACS analysis was performed with tetramer staining on

the x-axis and the CD8 staining on the y-axis. The amount of tetramer positive, CD8 positive cells are seen in the upper right quadrangle. The percentage of total cells being both tetramer and CD8 positive is calculated to the right of the frame and is given directly in the upper right quadrangle.

5

	T cells	Complexes	% tetramer+ and CD8+
Fig. 11A	relevant	relevant	53%
Fig. 11B	relevant	irrelevant	2%
Fig. 11C	irrelevant	relevant	1%
Fig. 11D	irrelevant	irrelevant	3%

10

EXAMPLES

15 ## Materials and methods

Urea, phenylmethylsulfonyl fluoride (PMSF), isopropyl-b-D thiogalactidase (IPTG), bicinchoninic acid solution (BCA) and tris[hydroxymethyl]aminomethane (tris) were purchased from Sigma. Sephadex G50 and Q-sepharose fast flow anion-exchange material were
20 purchased from Pharmacia, Sweden

Production of human and murine MHC class I heavy chains.

Recombinant HLA-A*0201 heavy chain (1-275) in XA90 cells was a kind gift from Drs. Wiley and Garboczi. XA90 cells from an over night culture were inoculated and grown at
25 37°C for production in 200 ml Luria-Bertani medium in 100 µg/ml ampicillin.

Recombinant H-2D^b (1-276) in pGMT7 vector (a pET derivative) was a kind gift from Dr Gallimore. The H-2D^b containing plasmid was transformed into Escherichia coli strain BL21(DE3) (Novagen) and grown at 37°C in 200 ml Luria-Bertani medium containing 100 µg/ml ampicillin. The cells from an over night culture were inoculated and grown at 37°C
30 in 200 ml Luria-Bertani medium in 100 µg/ml ampicillin for production.

Protein expression was induced at midlog phase (A600 = 0.6) with 0.4 mM isopropyl-b-d-thiogalactosidase (IPTG). The cells were harvested by centrifugation after 3 hours. The

cells were suspended in 10 ml. 20 mM tris buffer, pH 8.0 and 1 mM EDTA. and stored at -20°C.

Isolation of inclusion bodies and purification of recombinant HLA-A2*01 class I heavy chains.

5   Frozen cell preparations (from 200 ml cultures) were ruptured by sonication in 10 ml 20 mM tris, pH 8 with lyzosome (100 µg/ml), EDTA (1 mM), PMSF (50 µg/ml) and incubated 20 min. at room temperature. Subsequently, DNAse (10 µg/ml) and MgCl (10 mM) were added. After clearance, the inclusion bodies were partially purified by centrifugation in 15 min. at 10,000 g. Pellets containing inclusion bodies were washed 3 times in the tris

10 buffer. The pellets were finally solubilised by a 2 hour incubation in 3 ml 8 M urea, 20 mM tris, pH 8.0 with PMSF and EDTA at 4°C. Insoluble material was removed by centrifuga-tion. The supernatant was harvested and passed over 0.22 um filters before storage at -80°C. These preparations contained HLA-A*0201 class I heavy chains with a purity about 60 - 80% estimated from SDS-PAGE. The HLA-A*0201 class I heavy chains were purified

15 using a anion-exchange (Fast Flow, Pharmacia) column (1 x 25 cm). Preparations with partially purified heavy chains were diluted 5 fold with 8 M urea, 20 mM tris, pH 8.0 and applied the ion-exchange matrix. The column was washed with 20 ml 8 M urea, 20 mM tris, pH 8.0.and proteins were eluded in a gradient of 0 to 500 mM NaCl in 8 M urea, 20 mM tris, pH 8.0 buffer. Eluded proteins were monitored by BCA protein determination,

20 SDS-PAGE analysis and peptide binding capacity. Fractions containing highly purified monomer heavy chain proteins and with high capacity of peptide binding were pooled and stored at -80°C.

III.4 Purification of b2m and monoclonal antibodies.

Recombinant human and mouse b2m was produced and purified as described previously

25 (Pedersen et al., 1995). The monoclonal antibodies, W6/32 (α-HLA class I), BB7.2 (α-HLA- HLA-A*0201), 11-4.1 (α-Kk), S13-29 (α-Kk), 28-14-8S (α-H-2D^b), B22-249 (α-H-2D^b), were produced as ascites and purified by protein A chromatography (most of these hybridomas are from ATCC)

Radio-iodination of b2m and of peptide.

30 HLA-A*0201 and H-2D^b specific peptides were purified by reverse phase HPLC chroma-tography and lyophilised. The peptide (1-2 µg) was radiolabelled to a specific activity of 60

mCi/μg as previously described (Olsen et al., 1994). The fraction of labeled peptide bind-able to recombinant or native MHC class I was routinely 80%.

Electrophoresis

One-dimensional mini-slap SDS-polyacrylamide gel electrophoresis (PAGE) was per-
5   formed in homogeneous polyacrylamide gels (15%). Samples were boiled in Laemmli sample buffer with or without 50 mM DTT prior to SDS-PAGE analysis. Proteins were stained with Coomassie Blue R-250.

Peptide binding to recombinant HLA-A*201 class I molecules (tracer binding).

Denatured recombinant HLA-A*0201 heavy chains from the anion exchange purification
10 were tested for ability of peptide binding in the presence of b2m. Binding of peptide to re-combinant heavy chains was conducted essential as a conventional folding by dilution as-say (Garboczi et al,1992) - except that the amount of radiolabelled peptide was in tracer amount i.e. about 2 nM. Typically,1 microliter of denatured heavy chain solution from frac-tions of ion-exchange purification was diluted 100 fold in a folding buffer described below.
15 The reaction was incubated for at least 4 hours. The peptide binding was examined by Sephadex G25 spun column chromatography (Buus et al., 1995). All experiments have been conducted two or more times. Optimizing the binding of peptides to recombinant H-$2D^b$ and HLA-A*0201 revealed an optimal folding buffer consisting of 20 mM tris pH 7, 150 mM NaCl, 1 mM EDTA, 50 μg/ml PMSF, 1 μM b2m and 1-2 nM tracer peptide. It is
20 noteworthy that classical folding agents such as L-arginine and bacterial chaperonins such as GRO-EL/ES had a negative impact on the peptide binding (data not shown). Normal ranges of GSH/GSSG concentrations e.g. 1.8 mM/0.2 mM) had no effect on the peptide interaction. Peptide binding analysis in a temperature range from 4°C to 37°C in-dicated optimal binding at 18°C. Peptide binding analysis in a range of pH 5.5 to 9 indi-
25 cated an optimal binding at pH 6.5 - 7.5. Kinetic studies showed that tracer peptide bind-ing equilibrium was established after 4 hours incubation at 18°C.

Generation of functional HLA-A*201 class I molecules (scaled-up folding).

Denatured and purified HLA-A*201 heavy chain preparations (300 - 600 μg/ml) were folded by 100 fold dilution in a folding buffer (se above) to which peptide was added to a
30 concentration of about 10 μM. The reaction was concentrated 10-20 fold at 4°C using Amicon filter with a cut-off of about 10 kD. The concentrate was incubated at 4°C for 1

hour and centrifuged 15 min. at 15,000 g. Supernatants were concentrated further using Centricon units with a cut-off of about 3 kD at 4°C. Centrifugation was repeated and supernatants were applied to size-exclusion chromatography (Sephadex G50) to exclude free b2m and peptide. Fractions containing functional HLA-A*201 class I were pooled and

5 concentrated to a final concentration of about 1 mg/ml.

Results

Expression and purification of denatured recombinant HLA-A*201 heavy chain.

Recombinant HLA-A*0201 and H-2D^b were expressed and partially purified essentially as described by Garboczi et al. (Garboczi et al., 1992). The cells were induced with 0.4 mM

10 IPTG at a cell density about 0.6 and incubated for 3 hours at 37°C. The electrophoretic mobility of boiled and reduced samples with and without IPTG were analyzed in 15% SDS-PAGE gels. Yields of recombinant HLA-A*0201 were estimated to be about 40-50 mg/L culture corresponding to the predominating protein band of about 33 kD in figure 1, lane 3.

15 To isolate the inclusion bodies, the cells were ruptured by sonication. The cells expressing recombinant heavy chains (from a 200 ml culture) were centrifuged and pellets re-solubilised in buffers containing 20 mM tris, pH 8, lysozyme, PMSF and EDTA. Subsequently, DNAse and MgCl were added. After clearance of the solution (20-30 min. at 22°C) was centrifuged to pellet the inclusion bodies. Pellet was washed 3 times in tris buffer pH 8

20 and finally resolubilized in 8 M urea and stored at -80°C.

The partially purified proteins from inclusion bodies were further fractionated, using anion ion-exchange chromatography. Reasons for this purification step is to enrich the preparation for recombinant HLA-A*0201 molecules of high folding efficiently and to avoid heterogeneity (minor contaminants, minor enzymatic changes of the heavy chains in the bacte-

25 ria etc.)

Monomer recombinant HLA-A*0201 and H-2D^b were eluted in a gradient (0-500 mM) NaCl. Recombinant HLA-A*0201 and H-2D^b heavy chain monomers eluted at about 200 mM and 350 mM NaCl, respectively. The purity, concentration and functionality of the purified heavy chains were analyzed in SDS-PAGE (figure 1, lane 4 and figure 2), and by

30 BCA-protein determination assays and by tracer peptide binding analysis (figure 4).

Anion-exchange purification and non-reducing SDS-PAGE analysis showed that majority, if not all, of the recombinant HLA-A*0201 protein, solubilised from isolated inclusion bodies, were oxidized i.e. contained di-sulphide bridges. As shown in figure 2 the eluted recombinant HLA-A*0201 heavy chains migrated as two distinct bands (hereafter called 2a

5   and 3, respectively) in non-reducing SDS-PAGE analysis. We could not separate protein 2a from protein 3 using size-exclusion, ion-exchange or hydrophobic chromatography. Under reducing conditions, the two HLA-A*0201 heavy chains co-migrates slowly as one single protein band-called band 1 (figure 3, compare lane 2 and 3 with lane 4). This band correspond to the dominating HLA-A*0201 protein band in figure 1 (lane 3). In conclusion

10 the proteins appears packed in inclusion bodies with intact di-sulphur bridges leading to at least two distinct monomers. Folding experiments as described below, clearly shows that only protein band 2a folds into a functional recombinant HLA-A*0201 complex (figure 3, compare lane 1 and 2. Protein band 3 misfold and aggregates during a folding by dilution process. Presumably, protein band 2a contain correct di-sulphur bridge(s) whereas pro-

15 tein band 3 is incorrect. Also, chemical cross linking of radiolabelled peptide to recombinant HLA-A*0201 heavy chain identified the protein band 2a as the peptide receptor (data not shown).

The fast migration of proteins packed in inclusion bodies seems to be a general phenomenon. We have observed faster migration of widely different proteins as human b2m,

20 domains of the gamma and epsilon chains of the CD3 complex proteins, H2-K^k, MHC class II Alfa and beta chains in non-reducing SDS-PAGE analysis. Also recombinant H-2D^b molecules migrates with higher speed under non-reducing conditions (data not shown). In comparison with HLA-A*0201 heavy chains, the non-reduced H-2D^b heavy chains migrates as one single fast migrating band which migrates slower in reducing

25 SDS-PAGE analysis (data not shown).

Binding of radiolabelled peptides to partially purified denatured recombinant HLA-A*201 heavy chains.

Recombinant heavy chains fractionated by anion exchange chromatography were tested for their ability to bind radiolabelled specific peptides added during a folding by dilution

30 process (see materials and methods). As shown in (figure 4) there is a good correlation between appearance of monomer recombinant HLA-A*0201 heavy chain monomers (bands 2a and 3) and the ability of peptide binding. Purified heavy chains from urea preparation with reducing agent (more than 0.1 mM DTT) do not bind peptides after bio-

chemical purification even in presence of GSH/GSSG. Thus, the ability of peptide binding is related to heavy chains with pre-formed di-sulphide bridges.

Both recombinant HLA-A*0201 and recombinant H-2D^b heavy chains bound specific radioligand which could be inhibited by specific peptides, but not by non-binding peptides.

5 The recombinant HLA-A*0201 in complex with radiolabelled peptides could be precipitated with specific antibodies against HLA-A*0201 but not with antibodies against H2-Kk and H-2D^b molecules, indicating the generation of correct HLA-A*0201 (figure 5).

Binding of radiolabelled peptides to highly purified denatured recombinant HLA-A2*201 heavy chains.

10 Generation of functional MHC class I from a denatured state of heavy chains with pre-formed di-sulphur bridges was evaluated using radiolabelled peptide and radiolabelled b2m. Fully purified HLA-A*0201 heavy chains corresponding to protein band 2a were obtained through denaturation of functional recombinant HLA-A*0201 using 8 M urea. The denatured proteins were fractionated by size exclusion chromatography (Sephadex G50)

15 in a 20 mM tris buffer pH 8 with 8 M urea and the heavy chain was harvested in the void volume.

Binding of radiolabelled ligands (peptide or b2m) were done by dilution in folding buffer as described above. A dose response (figure 6) shows very efficient and sensitive binding of radiolabelled peptide to the denatured heavy chain during the folding process. In compari-

20 son, conventional affinity purified MHC class I molecules requires 10 to 50 fold higher concentration to bind similar amounts of peptide.

The high efficiency of peptide binding was analyzed in inhibition assay and by Scatchard analysis. As shown in figure 7, the recombinant HLA-A*0201 only interact with specific peptides. The Scatchard analysis (figure 7, insert) revealed a simple straight Scatchard

25 plot and an affinity equilibrium constant about 30 nM. Importantly, the fraction of active receptor was about 80 -90 % of the imput protein. Thus, the HLA-A*201 heavy chain was fully active without being preoccupied by peptides from the bacteria or derived from any of the subsequent handling steps. In comparison, conventional affinity purified MHC class I preparations are preoccupied with peptide having a limited number of active receptors,

30 typically 2%.

The peptide binding was very much dependent on b2m. As shown in figure 8, increasing doses of b2m added to the binding reaction facilitates binding of radiolabelled peptide. Ab-

sence of b2m completely prevented peptide binding to the heavy chain. Also the reverse reaction i.e. b2m binding to heavy chain show some dependence on the presence of specific peptides (figure 9). The peptide is however not an absolute requirement as b2m does bind,. albeit with a lower affinity, to the heavy chain in the absence of peptide.

5   The effect of peptides on b2m interaction was further analyzed in kinetic studies. Dissociation rates of HLA-A201 complexes consisting of radiolabelled b2m and heavy chains in the presence or absence of specific peptides were analyzed. Hetero-dimer complexes i.e. generated and maintained in absence of specific peptides dissociated rapidly with a half-life about 4 hours at 37°C. Trimeric peptide-b2m- heavy chain complexes i.e. generated

10 and maintained in presence of peptides were in comparison stable with a half-life about 14 hours. Thus peptides stabilizes the b2m binding. We conclude that a HLA-A*201 complex is generated through a primary interaction between the denatured heavy chain and b2m. The interaction generates a hetero-dimer, which express a high affine peptide binding site. Peptides in their turn increase the affinity of bound b2m molecules resulting in

15 stable functional HLA-A*201 complexes.

Generation of functional HLA-A*0201.

Fractions with high capacity of peptide binding (about 15 ml corresponding to 60-70% of the fractions with monomer heavy chain) were harvested and pooled for generation (folding) of functional HLA-A*0201 molecules. Recombinant HLA-A*0201 heavy chains were

20 diluted in folding buffer as described above. 500 µg, in 1 ml, partially purified heavy chain (corresponding to about 13 ml bacterial culture) was added to 99 ml buffer and immediately concentrated using Amicon filters with a cut off about 10 kD. 5-10 ml concentrate (obtained within 1 hour) was incubated for 1 hour at 4°C before centrifugation at 15,000g to remove aggregates. The supernatant was harvested and further concentrated to about

25 150-200 µl using centricon units with cut off about 3 kD. After additional centrifugation the folded HLA-A*0201 was purified using size exclusion chromatography (G50) which retain free b2m and peptides. Fractions containing the assembled HLA-A*0201 were pooled and concentrated using centricon to a final concentration about 1 mg/ml. The folding efficiency was about 40 - 50% calculated from the amount of added denatured protein. The yield of

30 functional HLA-A*0201, through the entire process correspond to about 10 mg functional HLA-A*0201 /L bacterial culture. The whole process of folding and purification can be conducted within 24 hours. Notice that folding was without addition of conventional agents as L-arginine and GSH/GSSG. The former inhibits folding of denatured HLA-A*0201

heavy chains with pre-formed disulfide bridges. The latter do not effect the outcome. The folded recombinant HLA-A*0201 molecules have routinely been tested in SDS-PAGE (figure 3) and for reactivity with specific antibodies (figure 5). Recombinant H-2D^b complexes generated by this procedure using biotinylated b2m was recently further assembled in oli-

5 gomeric complexes using streptavidin. The oligomerized ("tetramers") H-2D^b complexes was used for FACS staining of specific T cells (figure 11) and for staining of T cells as assessed by confocal microscopy. The latter demonstrated specific binding and internalisation.

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CLAIMS

1. A process of producing a functional immunoglobulin superfamily protein, which has at least one disulphide bond when functional, the process comprising the steps of

5

(i)   providing a bacterial cell comprising a gene coding for the protein, the gene is expressible in said cell,

(ii)   cultivating the cell under conditions where the gene is expressed,

10

(iii)   isolating the protein from the cell without reducing it,

(iv)   subjecting the isolated protein to a folding treatment.

15   2. A process of producing a plurality of functional proteins including at least one from the immunoglobulin superfamily and where the plurality of functional proteins when functional has at least one intramolecular and/or one intermolecular disulphide bond, the process comprising the steps of

20   (i)   providing a bacterial cell comprising a plurality of genes coding for one protein each, all genes being expressible in said cell,

(ii)   cultivating the cell under conditions where the genes are expressed,

25   (iii)   isolating the proteins from the cell without reducing them,

(iv)   subjecting the isolated proteins to a folding treatment.

3. A process of producing a functional immunoglobulin superfamily protein, which has at least one disulphide bond when functional, the process comprising the steps of

30   least one disulphide bond when functional, the process comprising the steps of

(i)   providing a cell comprising a gene coding for the protein, the gene is expressible in said cell, the protein being expressed as an aggregate,

35   (ii)   cultivating the cell under conditions where the gene is expressed,

(iii)   isolating the protein aggregate from the cell without reducing it,

(iv)   subjecting the isolated protein to a folding treatment.

5

4. A process according to any of claims 1-3 wherein the yield of functional protein pro-
duced according to the process relatively to the yield of functional protein obtained under
essentially similar conditions but where step (iii) is performed under reducing conditions,
is increased by at least 10%, such as at least 20%, at least 40%, at least 50%, at least

10   70%, or at least 100%.

5. A process according to any of claims 1-4 wherein when step (iii) is performed under
non-reducing conditions, the speed of the process compared to when step (iii) is per-
formed under reducing conditions is at least 10% faster.

15

6. A process according to any of claims 1-5 wherein the purity of the functional protein
produced according to the process relatively to the purity of the resulting functional protein
obtained under essentially similar conditions but where step (iii) is performed under re-
ducing conditions, is increased by at least 10%.

20

7. A process according to any of claims 1-6 wherein the protein is an immunoglobulin su-
perfamily protein selected from the group consisting of antibodies, immunoglobulin vari-
able (V) regions, immunoglobulin constant (C) regions, immunoglobulin light chains, im-
munoglobulin heavy chains, CD1, CD2, CD3, Class I and Class II histocompatibility mole-

25   cules, $\beta_2$microglobulin (β_2m), lymphocyte function associated antigen-3 (LFA-3) and
FcγRIII, CD7, CD8, Thy-1 and Tp44 (CD28), T cell receptor, CD4, polyimmunoglobulin
receptor, neuronal cell adhesion molecule (NCAM), myelin associated glycoprotein
(MAG), P myelin protein, carcinoembryonic antigen (CEA), platelet derived growth factor
receptor (PDGFR), colony stimulating factor-1 receptor, $\alpha\beta$-glycoprotein, ICAM (intercel-

30   lular adhesion molecule), platelet and interleukins.

8. A process according to any of claims 1-7 wherein the immunoglobulin superfamily pro-
tein is a vertebrate, e.g. protein such as a human, a murine, a rat, a porcine, a bovine, or
an avian protein.

35

9. A process according to any of claims 1-8 wherein the immunoglobulin superfamily protein is a MHC.

10. A process according to claim 9 wherein the MHC protein is a human MHC.

5

11. A process according to claim 9 or 10 wherein the MHC protein is a MHC class I protein selected from the group consisting of a heavy chain, a heavy chain combined with a β_2m, and a functional mature MHC class I protein; or a MHC class II protein selected from the group consisting of an α/β dimer and an α/β dimer with a peptide.

10

12. A process according to any of claims 9-11 wherein the produced MHC protein is obtained as a peptide free MHC protein.

13. A process according to any of claims 7-12 whereby when the folding process is final-
15 ized at least 25% of the produced immunoglobulin superfamily protein is obtained in a functional form.

14. A process according to any of claims 1-13 wherein the protein in step (ii) is produced as inclusion bodies.

20

15. A process according to any of claims 1-14 wherein step (iii) is performed under non-reducing conditions without altering the redox state.

16. A process according to any of claims 1-15 wherein the process further comprises a
25 step wherein the isolated protein from step (iii) is subjected to a purification step before step (iv).

17. A process according to any of claims 1-16 wherein the folding treatment (iv) is performed in an aqueous medium and at least one buffer compound.

30

18. A process according to any of claims 1-17 wherein the folding treatment is performed essentially in the absence of reducing agents, such as DTT.

19. A process according to any of claims 1-18 wherein the expressed protein is located
35 intracellularly.

20. A process according to any of claims 1-19 wherein the expressed protein is located periplasmatically.

5   21. A process according to any of claims 1-20 wherein the expressed protein is translocated extracellularly.

22. A process according to any of claims 1-21 wherein the protein is expressed in a glycosylated form.

10

23. A process according to any of claims 1-22 wherein the protein is expressed in a phosphorylated form.

24. A process according to any of claims 1-23 wherein the protein is glycosylated or

15 phosphorylated *in vitro*.

25. A process according to any of claims 1-24 wherein the protein comprises no unpaired Cysteine residues.

20 26. A process according to any of claims 1-25 wherein the protein comprises 1 unpaired Cysteine residue.

27. A process according to any of claims 1-26 wherein the protein comprises at least 2, such as at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least

25 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or at least 20 Cysteine residues.

28. A process according to any of claims 1-27 wherein the protein comprises an even number of Cysteine residues.

30

29. A process according to any of claims 1-28 wherein the protein is having at most 20, such as at most 14, at most 10, at most 8, at most 5, at most 4, at most 3, or at most 2 Cysteine residues.

30. A process according to any of claims 1-29 wherein the protein is capable of having at least 1, such as at least 2, at least 3, at least 4, at least 5, or at least 6 disulphide bonds.

31. A process according to any of claims 1-30 wherein the protein is capable of having at
5   most 20, such as at most 15, at most 10, at most 8, at most 5, at most 4, at most 3, or at most 2 disulphide bonds.

32. A process according to any of claims 3-31 wherein the cell is selected from the group consisting of a bacterial cell, a fungal cell, a yeast cell, an animal cell and a plant cell.
10
33. A process according to any of claims 1-32 wherein the cell is a bacterial cell selected from the group consisting of a gram positive bacterium and a gram negative bacterium.

34. A process according to claim 33 wherein the gram negative bacterium is *E. coli* in-
15 cluding a strain BL21 or a derivative thereof or a strain XA90 or a derivative thereof.

35. A process according to any of claims 1-34 wherein the cell is genetically modified to have a less reducing intracellular environment than a non-modified cell of the same strain.

20 36. A process according to any of claims 1-35 wherein the cell has been modified to have a reduced or lacking activity of a thioredoxin reductase or an enzyme having a similar ef-fect on the sulfhydryl reducing potential of the cytoplasm.

37. A process according to any of claims 1-36 wherein the modified cell is a *trxB*- mutant.
25
38. A process according to any of claims 1-37 wherein the gene is a derivative of a natu-rally occurring gene.

39. A process according to claim 38 wherein the derivative is obtained by substituting at
30 least one codon which is used more frequently by the host cell than the one originally pre-sent where the codon codes for the same amino acid.

40. A process according to any of claims 1-39 wherein the gene is under control of a regulatory DNA sequence not naturally associated with the gene.
35

41. A process according to any of claims 1-40 wherein the bacterial cell is transformed with an expression vector selected from the group consisting of pET vectors, e.g. T7 promoters.

5 42. A stable peptide free MHC protein obtainable by a process according to any of claims 1-41.

43. A kit comprising a MHC class I heavy chain and a β_2m allowing the recipient to produce and measure or detect a functional MHC class I protein to which a peptide, which is
10 capable of binding to said MHC class I protein, can be added leading to the generation of a functional MHC class I protein.

44. A kit according to claim 43 which comprises labelling of one or more MHC class I subunits (heavy chain, b2m and/or peptide) to measure or detect the generation of the MHC
15 class I protein.

45. A kit according to claim 43 and 44 wherein the measurement or detection system of the generated MHC class I protein is selected from the group of technologies consisting of radio-ligand, immuno-precipitation, ELISA, plasmon resonance, fluorescence polarization,
20 analytical ultracentrifugation, biochemical precipitation, ultrafiltration, chromatography and equilibrium dialysis.

46. A kit according to claims 43-45 which comprises an oligomerization of MHC proteins, such as two, three, four or more.
25
47. A kit according to claims 43-46 wherein a further reagent is added as a marker making the kit suitable for diagnostic purposes.

48. A kit according to claim 44-47 wherein the marker is selected from the group consist-
30 ing of biotin, fluorochromes, enzymes, chemiluminescense, and radioactive markers.

49. Use of a process according to any of claims 1-41 in the manufacturing of MHC.

50. Use of a stable empty MHC protein according to claim 42 in analysis of the effect of changing an amino acid in the MHC on the binding specificity of said MHC as assessed by an analysis using a peptide library approach be it a synthetic or recombinant library.
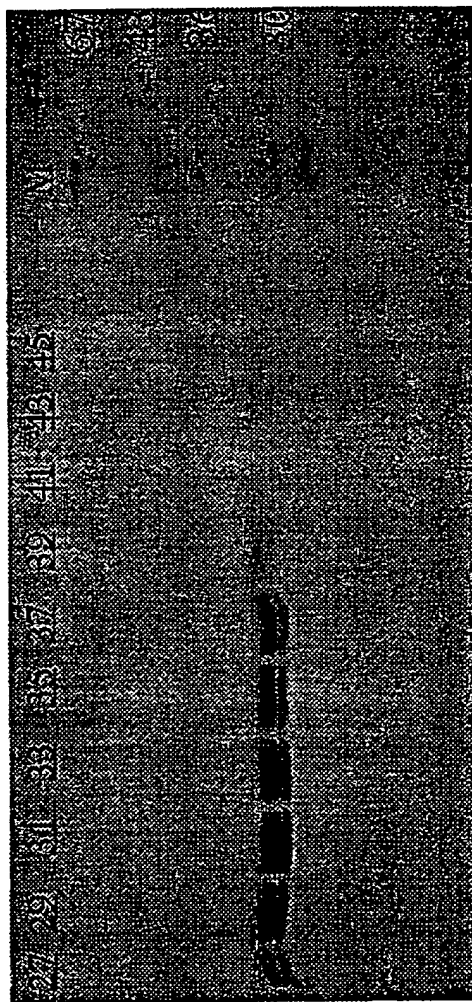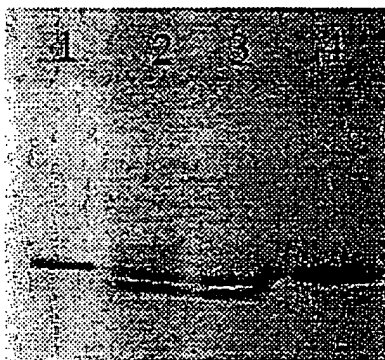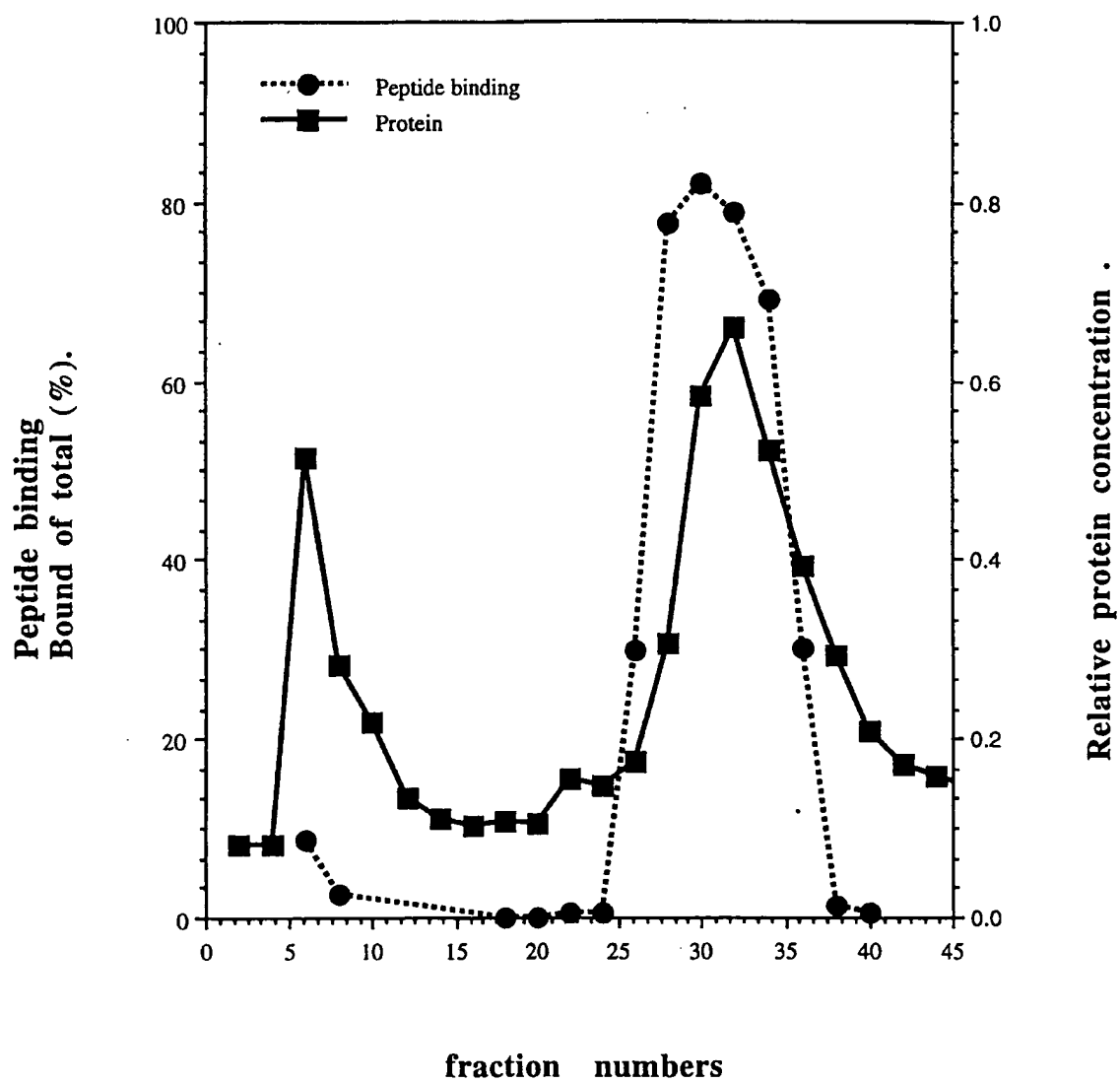
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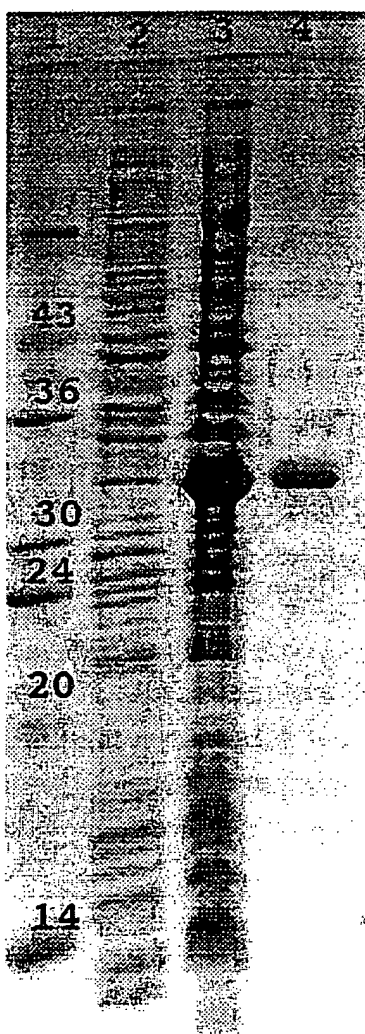
Fig. 1

Fig. 2

Fig. 3

4/12

fraction numbers

Fig. 4

Fig. 5

Binding of peptide to recombinant HLA-A*0201

Fig. 6

Fig. 7

b2M dependent peptide binding

Fig. 8

Binding of radiolabelled b2m to purified rA2.
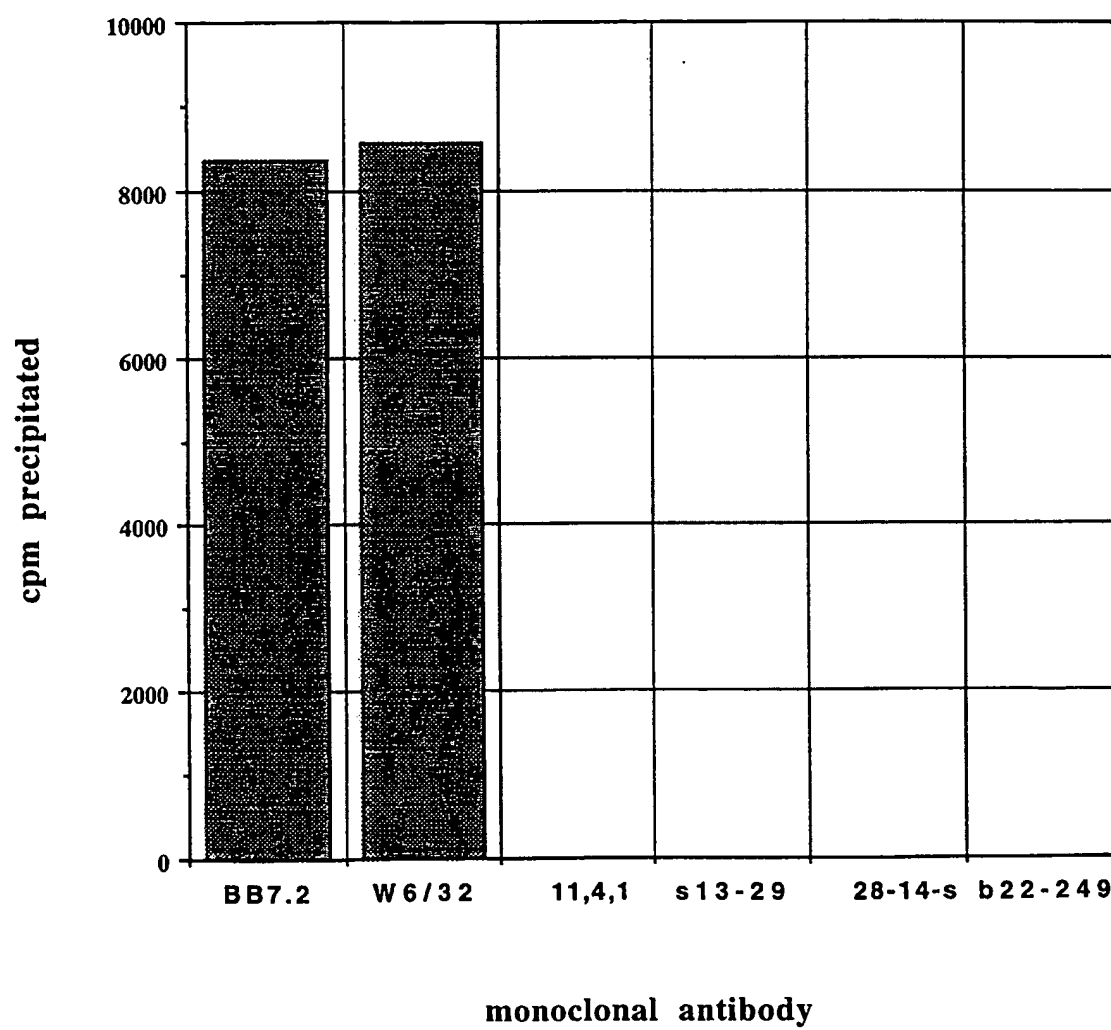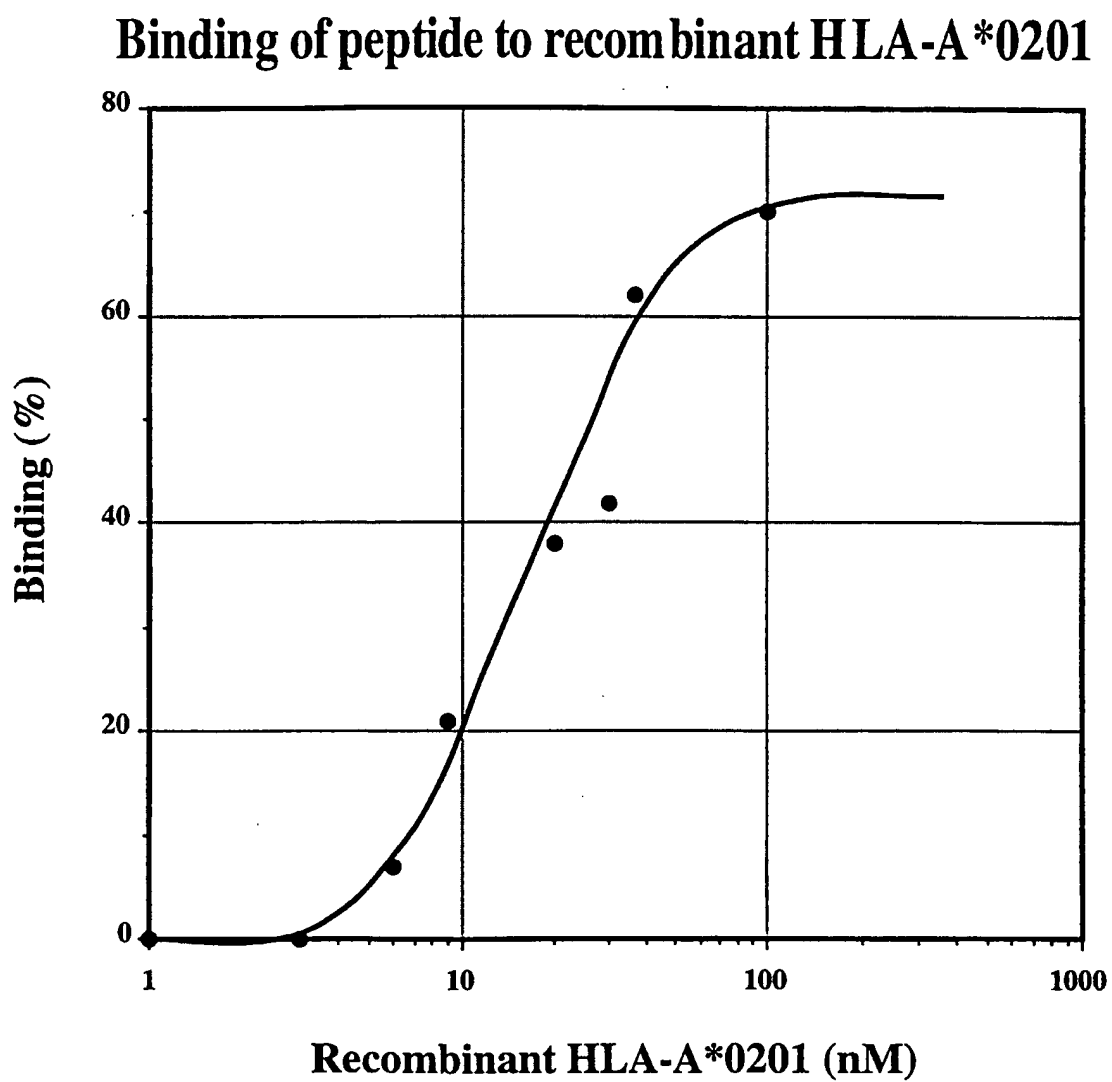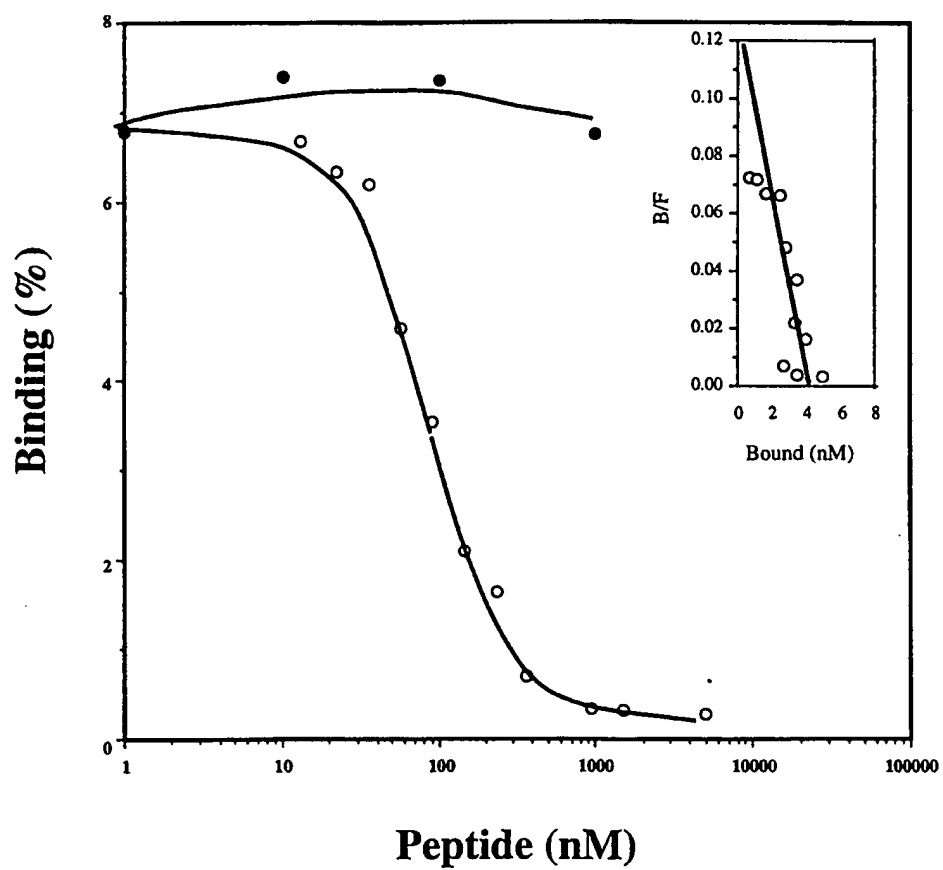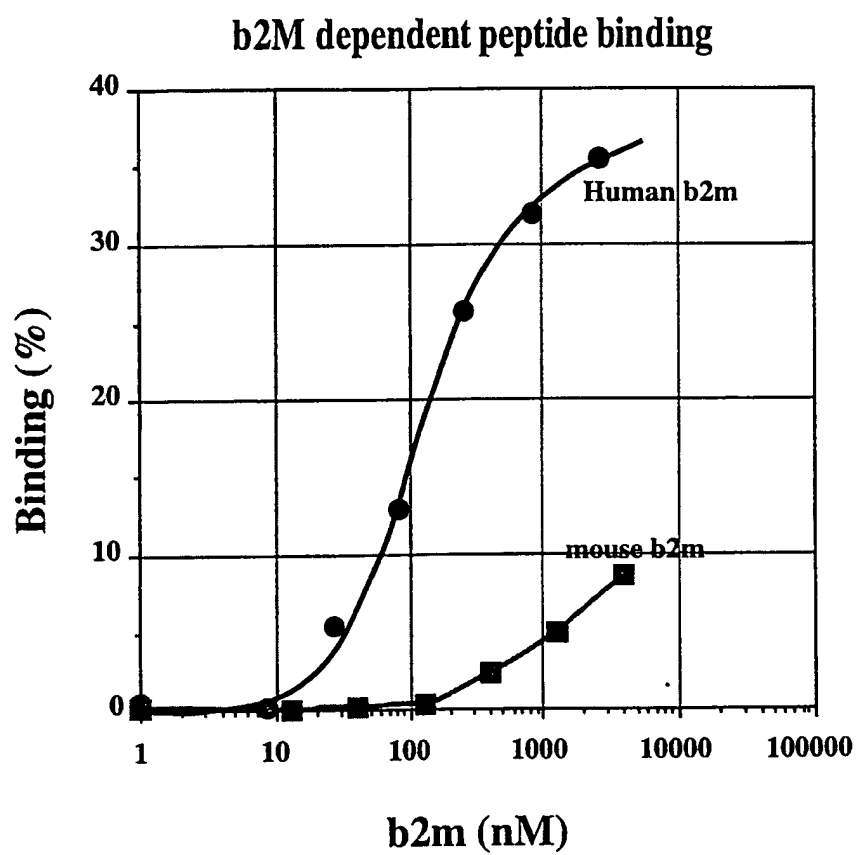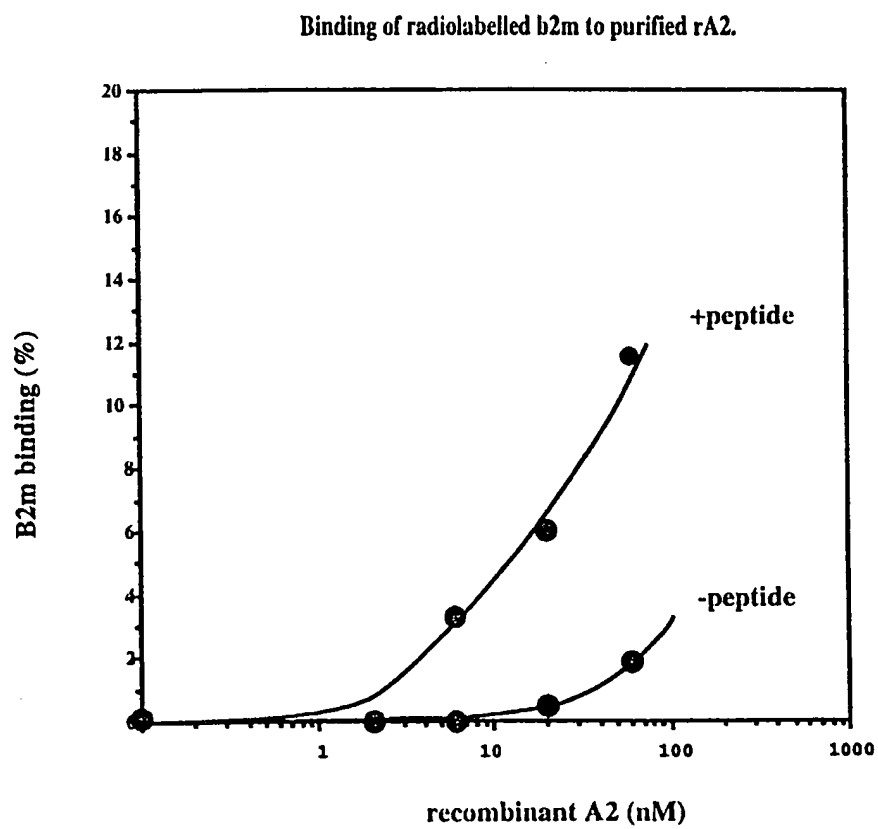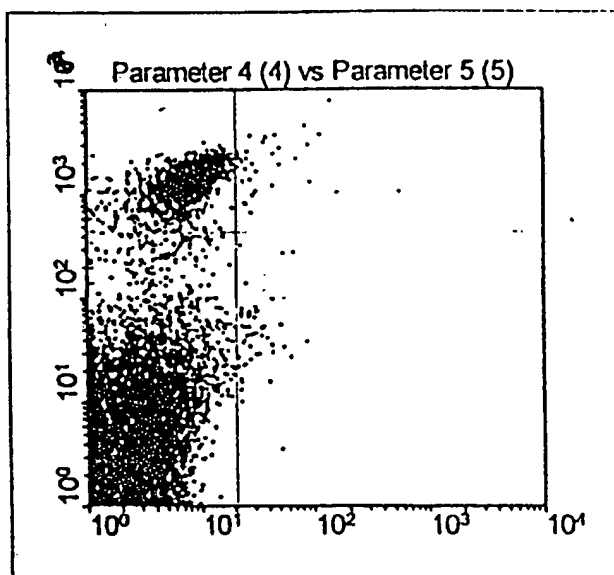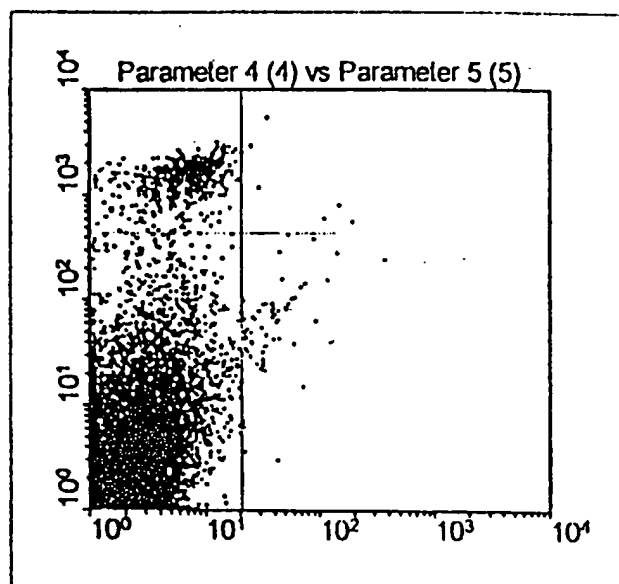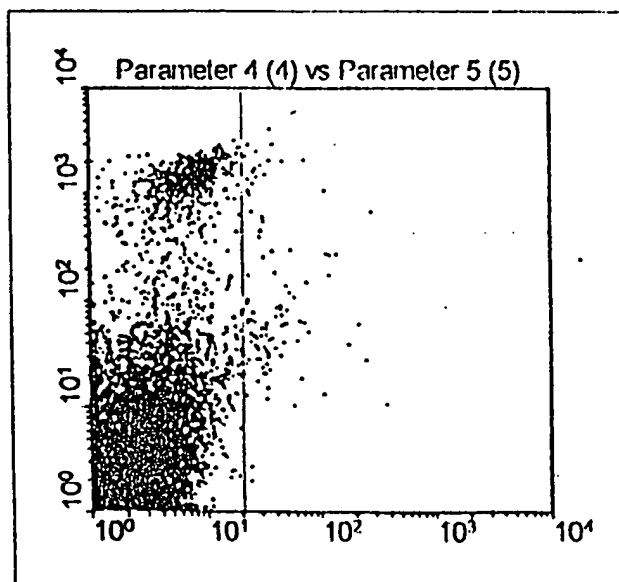
Fig. 9

Dissociation of labeled b2m from MHC class I.
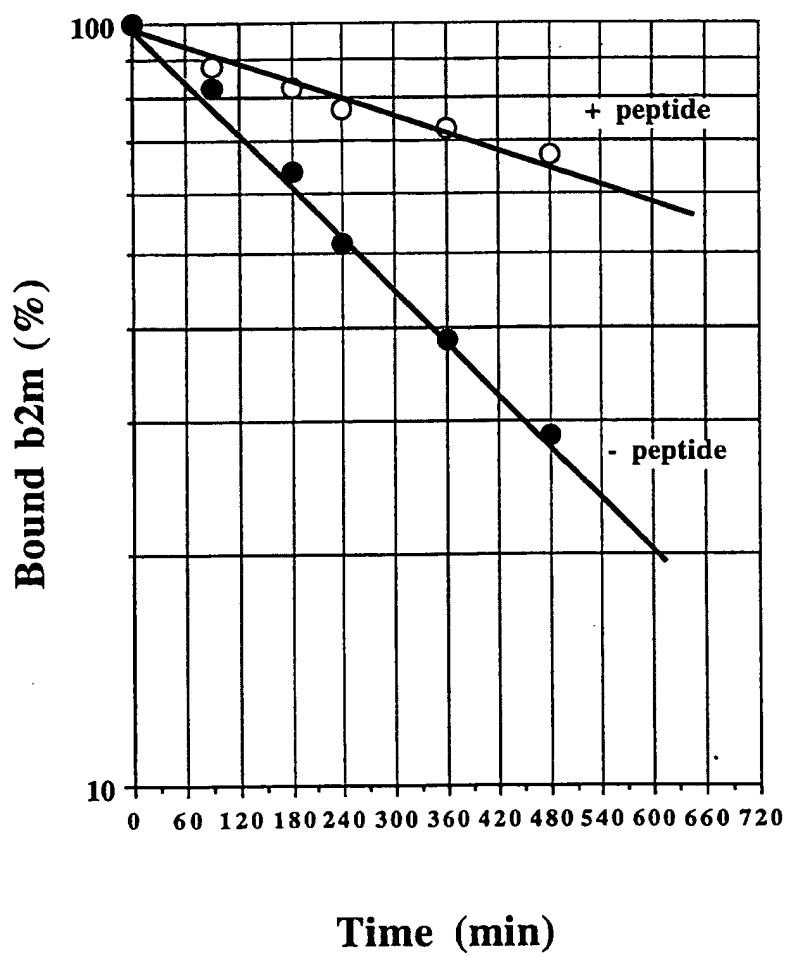
Fig. 10
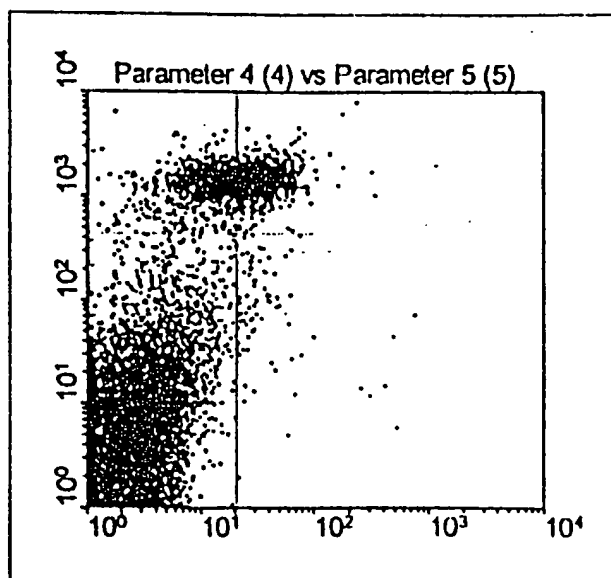
Fig. 11A

Fig. 11B

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Fig. 11C

Fig. 11D